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LIPASE VARIANTS

FIELD OF THE INVENTION

The present invention relates to novel lipase enzyme variants with improved properties, DNA constructs coding for the expression of said variants, host cells capable of expressing the variants from the DNA constructs, as well as a method of producing the variants by cultivation of said host cells. Furthermore, the present invention relates to a recombinant essentially pure Candida antarctica lipase and variants thereof as well as a DNA sequence encoding this lipase.

BACKGROUND OF THE INVENTION

A wide variety of lipases of microbial and mammalian origin are known. The amino acid sequence of many of these lipases have been elucidated and analyzed with respect to structural and functional elements important for their catalytic function, see, for instance, Winkler et al., 1990 and Schrag et al., 1991. It has been found that the lipase enzyme upon binding of a lipid substrate and activation undergoes a conformational change, which inter alia, results in an exposure of the active site to the substrate. This conformational change together with the presumed interaction between enzyme and substrate have been discussed by, inter alia, Brady et al., 1990, Brzozowski et al., 1991, Derewenda et al., 1992, and Derewenda and Derewenda, in press.

Based on the knowledge of the structure of a number of lipases, it has been possible to construct lipase variants having improved properties by use of recombinant DNA techniques. Thus, WO 92/05249 discloses the construction of certain lipase variants, in which the lipid contact zone has been modified so as to provide the variants with different substrate specificities and/or an improved accessibility of

the active site of the lipase to a lipid substrate. The modifications involve changing the electrostatic charge, hydrophobicity or the surface conformation of the lipid contact zone by way of amino acid substitutions.

Although the structural and functional relationship of lipases have been the subject of a number of studies as described in the above cited references, the research has mainly focused on the macroscopic characteristics of the lipases upon substrate binding and activation, whereas the identity of the amino acids actually involved in the substrate binding and catalytic activity has been discussed only to a lesser extent.

SUMMARY OF THE INVENTION

By sequence alignment analysis combined with analysis of the structure and activity of a number of lipases, the present inventors have now surprisingly found that the presence of certain amino acids, especially tryptophan, in a critical position of the lipase seems to be important for optimal catalytic activity.

It is consequently an object of the present invention to modify lipases which do not comprise such an amino acid residue, especially a tryptophan residue, in the critical position (which lipases in the present context are termed parent lipases) by replacing the amino acid residue located in this zone with a tryptophan residue so as to obtain variants having an increased specific activity.

More specifically, in one aspect the present invention relates to a lipase variant of a parent lipase comprising a

trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and, located in a critical position of a lipid contact zone of the lipase structure, an amino acid residue different from a tryptophan residue, which interacts with a lipid substrate at or during hydrolysis, in which lipase variant said amino acid residue has been replaced by a tryptophan residue so as to confer to the variant an increased specific activity as compared to that of the parent lipase.

In the present context, the term "trypsin-like" is intended to indicate that the parent lipase comprises a catalytic triad at the active site corresponding to that of trypsin, i.e. the amino acids Ser, His and one of Asp, Glu, Asn or Gln.

All lipases studied until now have been found to comprise at least one surface loop structure (also termed a lid or a flap) which covers the active serine when the lipase is in inactive form (an example of such a lipase is described by Brady et al., 1990). When the lipase is activated, the loop structure is shifted to expose the active site residues, creating a surface with increased surface hydrophobicity which interacts with the lipid substrate at or during hydrolysis. For the present purpose, this surface is termed the "lipid contact zone", intended to include amino acid residues located within or forming part of this surface. These residues may participate in lipase interaction with the substrate at or during hydrolysis where the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface. In the present context, the "critical position" of the lipase molecule is the position in the lipid contact zone of the lipase molecule, which is occupied by an amino acid residue which interacts with the lipid substrate and which is different from a tryptophan residue. Further

important parts of the activated lipase molecule are the binding pocket which constitutes the part of the lipid contact zone to which the lipid substrate binds before hydrolysis, and the hydrolysis pocket, in which the hydrolysis of triglycerides takes place. The three-dimensional structure of the lipid contact zone of an activated Humicola lanuginosa lipase molecule is illustrated in Fig. 1B of WO 92/05249.

In another aspect the present invention relates an C. antarctica lipase A which is essentially free from other C. antarctica substances and which comprises the amino acid sequence identified in Fig. 1 or a variant thereof which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of the C. antarctica lipase having the amino acid sequence shown in Fig. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the C. antarctica lipase A.

The C. antarctica lipase A of the invention has a number of desirable properties including a high thermostability and activity at acidic pH and may advantageously be produced by use of recombinant DNA techniques, e.g. using the procedures described below. Thus, the lipase A of the invention may be obtained in a higher purity and a higher amount than the C. antarctica lipase A purified from wild type C. antarctica which is described in WO 88/02775.

Furthermore, the present invention relates to a DNA sequence encoding the C. antarctica lipase A having the amino acid sequence identified in Fig. 1 or a modification of said DNA

sequence encoding a variant of the C. antarctica lipase A as defined above.

In the present context "C. antarctica lipase A" is used interchangeably with "lipase A" and the variant of the C. antarctica lipase A is termed "lipase A variant".

The present invention also relates to a DNA construct comprising a DNA sequence encoding a lipase variant as indicated above or a DNA sequence encoding the C. antarctica lipase A, a recombinant expression vector carrying said DNA construct, a cell transformed with the DNA construct or the expression vector, as well as a method of producing a lipase variant of the invention by culturing said cell under conditions conducive to the production of the lipase variant, after which the lipase variant is recovered from the culture.

It will be understood that the lipase variants of the present invention having an increased specific activity as compared to their parent lipases may be used for the same purposes as their parent lipases, advantageously in a lower amount due to their higher specific activity.

Accordingly, the present invention relates to the use of a lipase variant of the invention as a detergent enzyme; as a digestive enzyme; in ester hydrolysis, ester synthesis or interesterification; or the use of the lipase variant to avoid pitch trouble arising, e.g., in processes for preparing mechanical pulp and in paper-making processes using mechanical pulp.

DETAILED DISCLOSURE OF THE INVENTION

As indicated above, the present inventors have found that the presence of certain aromatic amino acids, especially tryptophan, located in the lipid contact zone of the lipase

molecule is important for optimal catalytic activity.

The importance of the presence of the tryptophan residue was found in connection with a study of mutants of a Humicola lanuginosa lipase which comprises a tryptophan residue at the critical position in the lipid contact zone, i.e. the amino acid number 89 in the amino acid sequence of the H. lanuginosa lipase published in EP 0 305 216. In the H. lanuginosa mutants this tryptophan residue was replaced by phenylalanine, tyrosine, histidine, isoleucine, glutamic acid and glycine, respectively. It was found that the specific activity of these mutants decreased (in the order indicated above) from 100% of the wild type lipase to about 10% of the phenylalanine mutant and down to about 2% for the glycine mutant.

While the critical position in some lipases is contemplated to be any position within the lipid contact zone, the critical position will normally be located in the binding pocket of the lipase molecule, and preferably in the hydrolysis pocket thereof. For most lipases it is believed that the critical amino acid residue is positioned on top of or in the proximity of the active site.

The amino acid residue occupying this position may be identified in any lipase by 1) sequence alignment studies in which the amino acid sequence of the lipase in question is aligned with the amino acid sequence of other lipases, in which the amino acid residue positioned on top of or in the proximity of the active serine has been identified, so as to identify the presumed position of said amino acid residue, and/or 2) an analysis of the three-dimensional structure of the lipase in question using standard display programmes such as INSIDE so as identify the amino acid sequence on top of or in the proximity of the active serine.

In some lipases the critical amino acid residue is located in the surface loop structure or in one or more of the surface loop structures if the lipase comprises more than one surface loop structure.

The amino acid residue present in the critical position, e.g. on top of or in the proximity of the active serine, is believed to be involved in a) stabilization of the tetrahedral intermediate formed from the lipase and the substrate during the activation of the lipase, and b) in the activation of the replacement of the lid region covering the active serine in the inactive enzyme. When tryptophan is present in this position, it is contemplated that optimal performance with respect to a) as well as b) above is obtained. Thus, it is believed that tryptophan gives rise to the formation of the most stable tetrahedral intermediate (which means a lowering of the activation energy needed for the catalysis to take place), and further improves the performance of the enzyme with respect to the activation of the lid opening which is essential for any catalysis to take place.

In connection with a) above it has been observed that the best acting lipase variants contain an unsaturated ring system in the side-chain. The far the biggest unsaturated system is tryptophan, then tyrosine, phenylalanine and histidine. These sidechains have a pi-electron system ("the unsaturation") that could be important for the proton transfer in the catalysis resulting in a lower activation energy for creating the tetrahedral intermediate where proton transfer has taken place from active site histidine to serine to the oxyanion hole created after lid activation and opening.

From the above theoretical explanation it will be understood that the optimal amino acid to be present in the critical position, e.g. on top of or in the proximity of the active

serine, is tryptophan. However, when the parent lipase is one which does not contain any aromatic amino acid residue or any amino acid residue with an unsaturated ring system in the side-chain in this position, such amino acids may advantageously be substituted into this position.

Thus, when the parent lipase, in the critical position, has an amino acid residue which does not comprise an unsaturated ring system in the side-chain, an amino acid residue having such an unsaturated ring-system, e.g. an aromatic amino acid (tryptophan, tyrosine or phenylalanine) or histidine may be substituted into the critical position. When the amino acid residue in the critical position of the parent lipase is histidine, it may advantageously be replaced by phenylalanine, tyrosine and most preferably tryptophan, when the amino acid residue is tyrosine, it may advantageously be replaced by phenylalanine and most preferably tryptophan, and when the amino acid residue is phenylalanine it may advantageously be replaced by tryptophan.

Although the critical position is normally considered to be constituted of only one amino acid residue it may be advantageous to replace two or more residues, preferably with a tryptophan residue as explained above, in order to obtain a further increased specific acitivity.

It is contemplated that it is possible to increase the specific activity of parent lipases which do not have a tryptophan residue in the critical position at least 2 times, such as at least 3 and preferably at least 4 or even 5, 6 or 7 times by modifications as diclosed herein.

It is contemplated that lipase variants as defined herein having an increased substrate specificity may be prepared on the basis of parent lipases of various origins. Thus, the parent lipase may be a microbial lipase or a mammalian lipa-

se.

When the parent lipase is a microbial lipase, it may be selected from yeast, e.g. Candida, lipases, bacterial, e.g. Pseudomonas, lipases or fungal, e.g. Humicola or Rhizomucor lipases.

One preferred lipase variant is one, in which the parent lipase is derived from a strain of Candida antarctica, in particular one in which the parent lipase is lipase A of C. antarctica, preferably the one which has the amino acid sequence shown in Fig. 1 or a lipase A variant thereof as defined herein. The lipase variant of this C. antarctica lipase A preferably has the amino acid sequence shown in Fig. 1 in which the phenylalanine 139 of the parent lipase has been replaced by a tryptophan residue. The construction of this variant and the analysis of the properties thereof is discussed in Example 3, 5 and 6.

A lipase variant of the invention may, as mentioned above, be prepared on the basis of a parent lipase derived from a strain of a Pseudomonas species, e.g. Ps. fragi. An example of a suitable Ps. fragi lipase which has an amino acid residue different from tryptophan positioned on top of or in the proximity of the active serine, is the one described by Aoyama et al., 1988. A lipase variant according to the present invention may be constructed by replacing the phenylalanine residue 29 in the amino acid sequence of said lipase shown in Fig. 2 by a tryptophan residue.

An example of a fungal lipase suitable as a parent lipase for the construction of a lipase variant of the invention is one derived from Rhizopus, especially from R. delemar or R. niveus, the amino acid sequence of which latter is disclosed in, e.g., JP 64-80290. In order to construct a lipase variant according to the present invention from this parent lipase,

the alanine residue at position 117 is to be replaced with a tryptophan residue. The sequence alignment of the R. niveus lipase sequence and an Rhizomucor miehei lipase sequence (containing a tryptophan residue) is illustrated in Fig. 3.

The present inventors have surprisingly found that non-pancreatic lipases such as gastric, lingual, or hepatic lipases have the common feature that the amino acid residue which has been identified to be the one located in the critical position of the lipase molecule, normally on top of or in the proximity of the active serine, is different from tryptophan. This is in contrast to pancreatic lipases which generally have been found to have a tryptophan residue in this position. Thus, in the present context, non-pancreatic mammalian lipases may advantageously be used as "parent lipases" for the construction of lipase variants of the invention.

Accordingly, lipase variants as disclosed herein which is of mammalian origin is advantageously prepared from a parent lipase of non-pancreatic, such as gastric, lingual or hepatic origin. Such mammalian lipases may be derived from humans, rats, mice, pigs, dogs or other mammals. Specific examples of such mammalian lipases includes a rat lingual lipase (Docherty et al., 1985), a rat hepatic lipase (Komaromy and Schotz, 1987), a human hepatic lipase (Datta et al., 1988), a human gastric lipase (Bodmer et al., 1987), and a Bio Salt Activated Lipase (BSAL) (Baba et al., 1991) all of which were analysed with respect to the critical position in the sequence alignment analysis illustrated in Fig. 4. The pancreatic lipases included in this sequence alignment study were a murine pancreatic lipase (Grusby et al., 1990), a porcine pancreatic lipase (Caro et al., 1981), a human pancreatic lipase (Lowe et al., 1989), and a canine pancreatic lipase (Mickel et al., 1989).

As mentioned above the present invention also relates to a C.

antarctica lipase A essentially free from other C. antarctica substances, which has the amino acid sequence shown in Fig. 1 or a variant therof which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of C. antarctica lipase A having the amino acid sequence shown in Fig. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the C. antarctica lipase A.

In the present context, the term "variant" is intended to indicate a lipase A variant which is derived from the C. antarctica lipase A having the amino acid sequence shown in Fig. 1, or a naturally occurring variant. Typically, the variant differ from the native lipase A by one or more amino acid residues, which may have been added or deleted from either or both of the N-terminal or C-terminal end of the lipase, inserted or deleted at one or more sites within the amino acid sequence of the lipase or substituted with one or more amino acid residues within, or at either or both ends of the amino acid sequence of the lipase.

Furthermore, the variant of the invention has one or more of the characterizing properties 1)-3) mentioned above. Property 1), i.e. the "lipase activity" of the variant may be determined using any known lipase assay, e.g. the Standard LU assay described in the Methods section below.

Property 2), i.e. the reactivity of the variant of the invention with an antibody raised against or reactive with at least one epitope of the C. antarctica lipase A having the

amino acid sequence shown in Fig. 1 below may be determined by polyclonal antibodies produced in a known manner, for instance by immunization of a rabbit with the C. antarctica lipase A of the invention. The antibody reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay.

Property 3) above, involving hybridization, may be performed using an oligonucleotide probe prepared on the basis of the full or partial cDNA sequence encoding the C. antarctica lipase A, the amino acid sequence of which is identified in Fig. 1, as a hybridization probe in a hybridization experiment carried out under standard hybridization conditions, e.g. 6 X SSC at 50°C. The nucleotide sequence on the basis of which the oligonucleotide probe is prepared is conveniently the DNA sequence shown in Fig. 1.

As stated above in a further aspect the present invention relates to a DNA sequence encoding C. antarctica lipase A having the amino acid sequence shown in Fig. 1 or a modification of said DNA sequence which encodes a variant of C. antarctica lipase A which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of the C. antarctica lipase A having the amino acid sequence shown in Fig. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the C. antarctica lipase A.

Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another

amino acid sequence of the encoded enzyme, but which may correspond to the codon usage of the host organism into which the DNA sequence is introduced or nucleotide substitutions which do give rise to a different amino acid sequence, without, however, impairing the above stated properties of the enzyme. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence and deletion of one or more nucleotides at either end of or within the sequence.

Methods of preparing lipase variants of the invention

Several methods for introducing mutations into genes are known in the art. After a brief discussion of cloning lipase-encoding DNA sequences, methods for generating mutations at specific sites within the lipase-encoding sequence will be discussed.

Cloning a DNA sequence encoding a lipase

The DNA sequence encoding a parent lipase or the C. antarctica lipase A may be isolated from any cell or microorganism producing the lipase in question by various methods, well known in the art. First a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the lipase to be studied. Then, if the amino acid sequence of the lipase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify lipase-encoding clones from a genomic library of bacterial DNA, or from a fungal cDNA library. Alternatively, a labelled oligonucleotide probe containing sequences homologous to lipase from another strain of bacteria or fungus could be used as a probe to identify lipase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying lipase-producing clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming lipase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for lipase. Those bacteria containing lipase-bearing plasmid will produce colonies surrounded by a halo of clear agar, due to digestion of the substrate by secreted lipase.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., The EMBO J. 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence, in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

Site-directed mutagenesis of the lipase-encoding sequence

Once a lipase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired

mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the lipase-encoding sequence, is created in a vector carrying the lipase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984, Biotechnology 2:646-639). U.S. Patent number 4,760,025, by Estell et al., issued July 26, 1988, discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette, however, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into lipase-encoding sequences is described in Nelson and Long, Analytical Biochemistry 180, 1989, pp. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Expression of lipase variants

According to the invention, a C. antarctica lipase A-coding sequence or a mutated lipase-coding sequence produced by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the

secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the lipase-coding sequence. For expression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant lipase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

According to one embodiment B. subtilis is transformed by an expression vector carrying the lipase A or the mutated DNA. If expression is to take place in a secreting microorganism such as B. subtilis a signal sequence may follow the translation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when is present.

In a currently preferred method of producing lipase A or lipase variants of the invention, a filamentous fungus is used as the host organism. The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of Aspergillus sp., such as A. niger, A. nidulans or A. oryzae. The use of A. oryzae in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

For expression of lipase variants in Aspergillus, the DNA

sequence coding for the lipase A or the lipase variant is preceded by a promoter. The promoter may be any DNA sequence exhibiting a strong transcriptional activity in Aspergillus and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α-amylase, A. niger acid stable α-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease or A. oryzae triose phosphate isomerase.

In particular when the host organism is A. oryzae, a preferred promoter for use in the process of the present invention is the A. oryzae TAKA amylase promoter as it exhibits a strong transcriptional activity in A. oryzae. The sequence of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may suitably be as described in EP 238 023.

To ensure secretion of the lipase A or the lipase variant from the host cell, the DNA sequence encoding the lipase variant may be preceded by a signal sequence which may be a naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an Aspergillus sp. amylase or glucoamylase, a gene encoding a Rhizomucor miehei lipase or protease, or a gene encoding a Humicola cellulase, xylanase or lipase. The signal sequence is preferably derived from the

gene encoding A. oryzae TAKA amylase, A. niger neutral α-amylase, A. niger acid-stable α-amylase or A. niger glucoamylase.

The medium used to culture the transformed host cells may be any conventional medium suitable for culturing Aspergillus cells. The transformants are usually stable and may be cultured in the absence of selection pressure. However, if the transformants are found to be unstable, a selection marker introduced into the cells may be used for selection.

The mature lipase protein secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

It will be understood that the lipase variants of the invention are contemplated to be active towards the same type of substrates as their parent lipases, with an improved specific activity. Thus, the lipase variants of the invention are contemplated to be useful for the same purposes as their parent lipases.

Accordingly, lipase variants of the invention prepared from a parent lipase useful as a detergent enzyme may be used as an active ingredient in a detergent additive or a detergent composition.

Another contemplated use of lipase variants of the invention, is as digestive enzymes, e.g. in the treatment of cystic fibrosis.

A third use of the lipase variants of the invention, especially variants of C. antarctica lipases are in lipase-catalysed processes such as in ester hydrolysis, ester synthesis and interesterification. The use of lipases in these processes is discussed in detail in WO 88/02775 (Novo Nordisk A/S), the content of which is incorporated herein by reference. Also the lipase variants of the invention may be used to avoid pitch trouble that arises in the production process for mechanical pulp or in a paper-making process using mechanical pulp, e.g. as described in PCT/DK92/00025 (Novo Nordisk A/S), the content of which is incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described in the following with reference to the appended drawings, in which

Fig. 1A illustrates the amino acid sequence of the mature C. antarctica lipase A,

Fig. 1b illustrates the amino acid sequence of the C. antarctica lipase A preproenzyme,

Fig. 1C illustrates the amino acid and nucleotide sequence of the C. antarctica lipase A of the invention,

Fig. 2 is the amino acid sequence of the Ps. fragi lipase described by Aoyama et al. 1988,

Fig. 3 is an amino acid sequence alignment of a R. niveus lipase sequence and an Rhizomucor miehei lipase sequence from which the critical position of the R. niveus lipase may be determined,

Fig. 4 illustrates the sequence alignment of a number of

mammalian pancreatic and non-pancreatic lipases, in which

1) is the amino acid sequence of the Bio Salt Activated Lipase described by Baba et al., 1991,

2) is the amino acid sequence of the rat lingual lipase described by Docherty et al., 1985,

3) is the amino acid sequence of the human gastric lipase described by Bodmer et al., 1987,

4) is the amino acid sequence of the dog pancreatic lipase described by Mickel et al., 1989,

5) is the amino acid sequence of the human pancreatic lipase described by Lowe et al., 1989,

6) is the amino acid sequence of the murine pancreatic lipase described by Grusby et al., 1990,

7) is the amino acid sequence of the porcine pancreatic lipase described by Caro et al., 1981,

8) is the amino acid sequence of the human hepatic lipase described by Datta et al., 1988, and

9) is the amino acid sequence of the rat hepatic lipase described by Komaromy et al., 1987,

Fig. 5 illustrates the N-terminal sequence of the lipase A from the C. antarctica strain LF 058, and the nucleotide sequences of the oligonucleotide probes NOR 438 and NOR 440 (see Example 1),

Fig. 6 illustrates the nucleotide sequence covering the open reading frame of the C. antarctica lipase A of the invention

(see Example 1),

Fig. 7 is a table showing the amino acid compositions of the C. antarctica lipase A of the invention and of a purfied wild type lipase A, and

Figs. 8 and 9 illustrate the scheme for the construction of the expression plasmid pMT1229 (see Example 1).

The present invention is further illustrated in the following examples which are not intended, in any way, to limit the scope of the invention as claimed.

GENERAL METHODS

Site-directed in vitro mutagenesis of lipase genes

The three different approaches described in WO 92/05249 may be used for introducing mutations into the lipase genes, i.e. the oligonucleotide site-directed mutagenesis which is described by Zoller & Smith, DNA, Vol. 3, No. 6, 479-488 (1984), the PCR method as described in Nelson & Long, Analytical Biochemistry, 180, 147-151 (1989), and the so-called "cassette mutagenesis" technique, in which a segment between two restriction sites of the lipase-encoding region is replaced by a synthetic DNA fragment carrying the desired mutation. Use of the latter technique is illustrated in Example 2.

Determination of lipase specific activity

Lipase activity was assayed using glycerine tributyrat as a substrate and gum-arabic as an emulsifier. 1 LU (Lipase Unit) is the amount of enzyme which liberates 1 μmol titratable butyric acid per minute at 30°C, pH 7.0. The lipase activity was assayed by pH-stat using Radiometer titrator VIT90,

Radiometer, Copenhagen. Further details of the assay are given in Novo Analytical Method AF 95/5, available on request.

EXAMPLE 1

Cloning of Candida antarctica lipase A

Chromosomal DNA of the C. antarctica strain LF058 was prepared by opening of frozen cells by grinding with quartz and subsequent extraction of DNA essentially as described by Yelton et al., (1984). The purified DNA was cut partially with Sau3A and, after agarose gel electrophoresis, fragments in the range of 3-9 kb were isolated. The sized Sau3A fragments were ligated into a BamH1-cut, dephosphorylated plasmid pBR322 (New England Biolabs). The ligation mix was transformed into the K12 restriction deficient E. coli MC1000 derivative MT172. Approximately 50,000 transformant E. coli colonies were obtained, 80% of which contained an insert of LF058 DNA.

Using standard colony hybridization techniques (Maniatis et al., 1982) the colonies were screened with the 32^{P}-phosphorylated oligonucleotide probe NOR 440. NOR 440 is a degenerated (64) 17 mer based on the N-terminal determined from mature C. antarctica lipase A (see Fig. 1). 34 colonies appeared positive after wash at low stringency (41°C and 6 x SSC). Plasmids were prepared from these colonies and Southern analyzed after restriction with BstN1. The probe for the Southern was either the NOR 440 probe used for the colony hybridization (see above) or a 32^{P}-labelled probe NOR 438. NOR 438 is an oligonucleotide (a guess mer) where, at 13 positions, a base has been chosen on the basis of codon use in yeasts and filamentous fungi. Only one plasmid, pMT1076, contained a band which hybridised both to NOR 440 at low stringency (see above) and to NOR 438 at a somewhat higher stringency (55°C

and 1 x SSC).

PMT1076 was restriction mapped and the DNA sequence determined by the Maxam-Gilbert method. The sequence covering the open reading frame is shown in Fig. 6. The open reading frame is seen to encode a putative signal sequence of 21 amino acids (according to the von Heine rules (von Heijne, G. (1986)) and furthermore a propeptide of 10 amino acids preceding the N-terminal of the mature lipase. The last two amino acids of the propeptide are Arg Arg, i.e. a typical cleavage site for endoproteolytic processing by enzymes of the S. cereviciae KEX-2 type. The amino acid composition of the mature protein encoded by the DNA sequence is in agreement with the amino acid composition determined for C. antarctica lipase A (Fig. 7).

Through a number of standard plasmid manipulations (Maniatis et al., 1982) illustrated in Figs. 8 and 9, the open reading frame of C. antarctica lipase A was placed in the correct orientation between the alpha-amylase promoter of A. oryzae and the glucoamylase transcription terminator of A. niger. The resulting expression plasmid pMT1229 was transformed into A. oryzae A1560 as described in EP 305,216. Transformants were isolated and grown as described in the above cited patents and the culture supernatants were analyzed for the presence of C. antarctica lipase A.

EXAMPLE 2

Construction of a plasmid expressing the F135W variant of Candida antarctica lipase A

A 246 bp BamHI/BssHII fragment was synthesized in vitro on the basis of the nucleotide sequence of pMT1229 using oligonucleotide primers 3116 and 3117 in a PCR reaction. The primer 3117 includes a BssHII restriction site and a mutation

in the 135 phe codon (TTC) to trp codon (TGG) which is marked with stars.

Oligonucleotide primer 3116 (F135W:710-730)
5'-CAG AAC GAG GCG GTG GCC GAC-3'

Oligonucleotide primer 3117 (F135W:1020-940)
5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT TCG TAG CCA GCG ATG AAG GCG GCT TTC* C*AG CCT TCG TG-3'

The PCR reaction was performed by mixing the following components and incubating the mixture in a HYBAID™ thermal reactor.

Template pMT1229	10 ng/µl	1 µl
H_2O		46.5µl
10 x PCR buffer		10 µl
2 mM dATP		10 µl
2 mM dTTP		10 µl
2 mM dCTP		10 µl
2 mM dGTP		10 µl
primer 3116	50.5 pmol/µl	1 µl
primer 3117	70.5 pmol/µl	1 µl
Taq polymerase		0.5µl
Parafin oil		50 µl

Step I	94°C	2 min.	1 cycle
Step II	94°C	30 sec.	
	50°C	30 sec.	30 cycle
	72°C	2 min.	
Step III	72°C	5 min.	1 cycle

The resulting 310 bp fragment was isolated from a 2% agarose gel after electrophoresis and digested with BamHI and BssHII restriction enzymes. The resulting 264 bp BamHI/BssHII fragment was likewise isolated from 2% agarose gel. This fragment was then ligated with pMT1229 BamHI/XbaI 0.3 kb
pMT1229 BssHII/SphI 0.5 kb

pMT1229 SphI/XbaI 5.0 kb

The ligated DNA was transformed into E. coli strain MT172. Transformants which contained correct inserts were selected and their DNA sequence was determined by use of Sequenase (United States Biochemical Corporation). The pME1178 plasmid contained a mutation in the amino acid position 135 (phe was mutated to trp).

pME1178 was cotransformed with pToC186 which included the amdS gene from A. nidulans as a selective marker into the Aspergillus oryzae A1560 strain. A. oryzae transformants were reisolated twice on selective plates and stable transformants were characterized by rocket immunoelectrophoresis, using anti-Candida lipase A antibody. Candida lipase A produced by the MEA65 strain was further analyzed for specific activity.

EXAMPLE 3

Construction of a plasmid expressing the F139W variant of Candida antarctica lipase A

A 246 bp BamHI/BssHII fragment was synthesized in vitro on the basis of the nucleotide sequence of the plasmid pMT1229 using oligonucleotide primers 3116 and 3826 in a PCR reaction. The primer 3826 includes a BssHII restriction site and a mutation in the 139 phe codon (TTC) to trp codon (TGG) which is marked with stars.

Oligonucleotide primer 3116 is shown in Example 2.

Oligonucleotide primer 3826 (F139W:1020-941)
5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT TCG TAG CCA GCG ATC* C*AG GCG GCT TTG AAG CCT TCG TG-3'

A PCR reaction was performed by the method described in Example 2. The 310 bp fragment was isolated from 2% agarose gel after electrophoresis and digested by BamHI and BssHII restriction enzymes. The resulting 264 bp BamHI/BssHII fragment was likewise isolated from 2% agarose gel. This fragment was then ligated with

pMT1229	BamHI/XbaI	0.3 kb
pMT1229	BssHII/SphI	0.5 kb
pMT1229	SphI/XbaI	5.0 kb

The ligated DNA was transformed into E. coli strain MT172. Transformants which contained correct inserts were selected and their DNA sequence was determined by use of Sequenase (United States Biochemical Corporation). The pME1229 plasmid contained a mutation in the amino acid position 139 (phe was mutated to trp).

pME1229 was cotransformed with pToC202 which included the amdS gene from A. nidulans as a selective marker into Aspergillus oryzae A 1560 strain. A oryzae transformants were reisolated twice on selective plates and enzyme activity of a stable transformant MEA155 was analyzed by using tributylene as a substrate as described in Example 5.

EXAMPLE 4

Construction of a plasmid expressing the F135W/F139W variant of Candida antarctica lipase A

A 246 bp BamHI/BssHII fragment was synthesized in vitro using oligonucleotide primers 3116 and 4224 by a PCR reaction. The primer 4224 includes a BssHII restriction site and mutations in the 135 and 139 codons (TTC) to trp codons (TGG) which are marked with stars.

The oligonucleotide primer 3116 is shown in Example 2.

Oligonucleotide primer 4224 (F135W:1020-941)
5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT TCG TAG CCA GCG ATC* C*AG GCG GCT TTC* C*AG CCT TCG TG-3'

PCR reaction was performed by using the method shown in Example 2. The 310 bp fragment was isolated from a 2% agarose gel after electrophoresis and digested with BamHI and BssHII restriction enzymes. The resulting 264 bp BamHI/BssHII fragment was likewise isolated from a 2% agarose gel. This fragment was then ligated with

pMT1229	BamHI/XbaI	0.3 kb
pMT1229	BssHII/SphI	0.5 kb
pMT1229	SphI/XbaI	5.0 kb

The ligated DNA was transformed into E. coli MT172. Transformants which contained inserts were selected and their DNA sequence was determined by use of Sequenase. The pME1230 plasmid contained two mutations in the amino acid positions 135 and 139 (phe was mutated to trp).

pME1230 was cotransformed with pToC90 which included the amdS gene from A. nidulans as a selective marker into Aspergillus oryzae A 1560 strain. A. oryzae transformants were reisolated twice on selective plates and enzyme activity of stable transformants were analyzed by using tributylene as a substrate as described in Example 5.

EXAMPLE 5

Purification of C. antarctica lipase A variants F139W and F135W/F139W and comparison of specific activity with their parent wild type C. antarctica lipase A

The lipase variants and the parent lipase produced as described in Examples 3, 4 and 1, respectively, were purified using

the following 4 step standard purification procedure.

Step 1: The fermentation broth containing the lipase and lipase variant, respectively, obtained by culturing the transformed A. oryzae cells described in Examples 1 and 3 above, was centrifuged, and the supernatant was adjusted to pH 7. Ionic strength was adjusted to 2 mSi. DEAE-Sephadex A-50 (Pharmacia) gel was swollen and equilibrated in 25 mM Tris acetate buffer pH 7. The fermentation supernatant was passed through DEAE-Sephadex A-50 on scintered glass funnel. The effluent containing lipase activity was collected and adjusted to 0.8 M ammonium acetate.

Step 2: An appropriate column was packed with TSK gel Butyl-Toyopearl 650 C and equilibrated with 0.8 M ammonium acetate. The effluent containing lipase activity was applied on the column. The bound material was eluted with water.

Step 3: The lipase-containing eluate was then applied on a Highperformance Q-Sepharose column. Lipase activity was collected as effluent. The lipases purified by this method were concentrated to an Optical Density of 1 at 280 nm.

The purity of the lipases was checked by SDS-PAGE showing one band with an molecular weight of about 45 kD. The lipase activity was determined by use of the method outlined above in the section "General methods".

The lipase activity of the parent wild type lipase was 300 LU/OD_{280} as compared to 1200 LU/OD_{280} for the lipase variant F139W. On the basis of OD_{280} absorption without correction for the inserted tryptophan, the specific activity of the mutant was at least four times higher with the assay used. The lipase activity of the lipase variant F135W/F139W was 1400 LU/OD_{280} (without correction for the two additional tryptophans).

EXAMPLE 6

Thermostability of Candida antarctica lipase A and the mutant F139W thereof

The thermostability of the C. antarctica lipase A and the C. antarctica lipase A variant, was examined by Differential Scanning Calorimetry (DSC) at different pH values. Using this technique, the thermal denaturation temperature, T_d, is determined by heating an enzyme solution at a constant programmed rate.

More specifically, the Differential Scanning Calorimeter, MC-2D, from MicroCal Inc. was used for the investigations. Enzyme solutions were prepared in 50 mM buffer solutions, cf. the tables below. The enzyme concentration ranged between 0.6 and 0.9 mg/ml, and a total volume of about 1.2 ml was used for each experiment. All samples were heated from 25°C to 90°C at a scan rate of 90°C/hr.

The results obtained from the analysis is shown in the table below:

C. ant. lipase A (WT)

pH	Buffer (50 mM)	Denaturation temperature[1)]
4.5	Acetate	96°C
5	Acetate	95°C
7	TRIS	93°C

C. ant. lipase A mutant (F139W)

pH	Buffer (50 mM)	Denaturation temperature[1)]
5	Acetate	84°C
7	TRIS	82°C

1) Temperature, at which approximately half the enzyme molecules present have been denatured thermally during heating

The above results show that the pH-optimum for the thermostability of C. antarctica lipase A and the F139W variant is unusually low and that both enzymes are very thermostable below pH 7. Within the investigated range the thermostability of both the Wild Type and the mutant F139W continues to

increase as pH is lowered. This makes both lipases very well suited for hydrolysis/synthesis at unusually high temperatures at relatively low pH values.

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CLAIMS

1. A lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and, located in a critical position of a lipid contact zone of the lipase structure, an amino acid residue different from a tryptophan residue, which interacts with a lipid substrate at or during hydrolysis, in which lipase variant said amino acid residue has been replaced by a tryptophan residue so as to confer to the variant an increased specific activity as compared to that of the parent lipase.

2. A lipase variant according to claim 1, in which said amino acid residue different from a tryptophan residue is located in the binding pocket of the lipase molecule.

3. A lipase variant according to claim 2, in which said amino acid residue different from a tryptophan residue is located in a hydrolysis pocket of the lipase molecule.

4. A lipase variant according to any of claims 1-3, wherein said amino acid residue different from a tryptophan residue is located on top of or in the proximity of the active serine.

5. A lipase variant according to any of claims 1-4, wherein said amino acid residue different from a tryptophan residue is present in a lid region of the lipase.

6. A lipase variant according to any of the preceding claims, in which said amino acid residue different from a tryptophan residue is a phenylalanine residue.

7. A lipase variant according to any of the preceding claims, wherein the parent lipase is selected from a microbial or a mammalian lipase.

8. A lipase variant according to claim 7, wherein the parent lipase is a yeast lipase.

9. A lipase variant according to claim 8, wherein the parent lipase is derived from a strain of Candida antarctica.

10. A lipase variant according to claim 9, wherein the parent lipase is lipase A of C. antarctica.

11. A lipase variant according to claim 7, which has the amino acid sequence shown in Fig. 1 in which the phenylalanine 139 of the parent lipase has been replaced by a tryptophan residue.

12. A lipase variant according to claim 4, in which the parent lipase is a bacterial lipase.

13. A lipase variant according to claim 12, wherein the parent lipase is derived from a strain of Pseudomonas.

14. A lipase variant according to claim 13, which is derived from a strain of Ps. fragi.

15. A lipase variant according to claim 14, which has the amino acid sequence shown in Fig. 2 in which the phenylalanine 29 of the parent lipase has been replaced by a tryptophan residue.

16. A lipase variant according to claim 7, wherein the parent lipase is a fungal lipase.

17. A lipase variant according to claim 7, wherein the parent

lipase is a human lipase, a murine lipase, a rat lipase or a canine lipase.

18. A C. antarctica lipase A essentially free from other substances from C. antarctica, which comprises the amino acid sequence shown in Fig. 1, or a variant of said lipase which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of C. antarctica lipase A having the amino acid sequence SEQ ID No. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the C. antarctica lipase A.

19. A DNA sequence encoding C. antarctica lipase A having the amino acid sequence shown in Fig. 1 or a modification of said DNA sequence which encodes a variant of C. antarctica lipase A which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of the C. antarctica lipase A having the amino acid sequence SEQ ID No. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the C. antarctica lipase A.

20. A DNA construct comprising a DNA sequence encoding a lipase variant according to any of claims 1-17 or C. antarc-

tica lipase A according to claim 18.

21. A recombinant expression vector which carries a DNA construct according to claim 20.

22. A cell which is transformed with a DNA construct according to claim 20 or a vector according to claim 21.

23. A cell according to claim 22 which is a fungal cell, e.g. belonging to the genus Aspergillus, such as A. niger, A. oryzae, or A. nidulans; a yeast cell, e.g. belonging to a strain of Saccharomyces, such as S. cerevisiae, or a methylotrophic yeast from the genera Hansenula, such as H. polymorpha, or Phichia, such as P. pastoris; or a bacterial cell, e.g. belonging to a strain of Bacillus, such as B. subtilis, or B. lentus, or to a strain of Escherichia, such as E. coli.

24. A method of producing a lipase variant according to any of claims 1-17, wherein a cell according to any of claims 21-23 is cultured under conditions conducive to the production of the lipase variant, and the lipase variant is subsequently recovered from the culture.

25. Use of a lipase variant according to any of claims 1-17 as an active ingredient in a detergent additive or a detergent composition.

26. Use of a lipase variant according to any of claims 1-17 or the C. antarctica lipase A or a variant thereof according to claim 18 in ester hydrolysis, ester synthesis or interesterification.

27. The use according to claim 260, in which the lipase variant is the lipase variant according to any of claims 9-11.

28. Use of a lipase variant according to any of claims 1-17

or the C. antarctica lipase A or a variant thereof according to claim 18 for avoiding pitch trouble in a process for the production of mechanical pulp or a paper-making process using mechanical pulp.

29. The use according to claim 28, in which the lipase variant is the lipase variant according to any of claims 9-11.

C.antarctica lipase A.
Sequence of mature enzyme

AALPNPYDDPFYTTPSNIGTFAKGQVIQSRKVPTDIGNANNAASFQLQYRTTNTQNEAVADVATVW
IPAKPASPPKIFSYQVYEDATALDCAPSYSYLTGLDQPNKVTAVLDTPIIIGWALQQGYYVVSSDH
EGFKAAFIAGYEEGMAILDGIRALKNYQNLPSDSKVALEGYSGGAHATVWATSLAESYAPELNIVG
ASHGGTPVSAKDTFTFLNGGPFAGFALAGVSGLSLAHPDMESFIEARLNAKGQRTLKQIRGRGFCL
PQVVLTYPFLNVFSLVNDTNLLNEAPIASILKQETVVQAEASYTVSVPKFPRFIWHAIPDEIVPYQ
PAATYVKEQCAKGANINFSPYPIAEHLTAEIFGLVPSLWFIKQAFDGTTPKVICGTPIPAIAGITT
PSADQVLGSDLANQLRSLDGKQSAFGKPFGPITPP

Fig. 1A

C. antarctica lipase A.
Sequence of preproenzyme

MRVSLRSITSLLAAATAAVLAAPAAETLDRRAALPNPYDDPFYTTPSNIGTFAKGQVIQSRKVPTD
IGNANNAASFQLQYRTTNTQNEAVADVATVWIPAKPASPPKIFSYQVYEDATALDCAPSYSYLTGL
DQPNKVTAVLDTPIIIGWALQQGYYVVSSDHEGFKAAFIAGYEEGMAILDGIRALKNYQNLPSDSK
VALEGYSGGAHATVWATSLAESYAPELNIVGASHGGTPVSAKDTFTFLNGGPFAGFALAGVSGLSL
AHPDMESFIEARLNAKGQRTLKQIRGRGFCLPQVVLTYPFLNVFSLVNDTNLLNEAPIASILKQET
VVQAEASYTVSVPKFPRFIWHAIPDEIVPYQPAATYVKEQCAKGANINFSPYPIAEHLTAEIFGLV
PSLWFIKQAFDGTTPKVICGTPIPAIAGITTPSADQVLGSDLANQLRSLDGKQSAFGKPFGPITPP

Fig. 1B

Prepro C.antarctica lipase A -> 1-phase Translation

DNA sequence 1386 b.p. ATGCGAGTGTCC ... ATCACACCACCT linear

```
1/1                                                 31/11
ATG CGA GTG TCC TTG CGC TCC ATC ACG TCG CTG CTT GCG GCG GCA ACG GCG GCT GTG CTC
Met arg val ser leu arg ser ile thr ser leu leu ala ala ala thr ala ala val leu
61/21                                               91/31
GCG GCT CCG GCG GCC GAG ACG CTG GAC CGA CGG GCG GCG CTG CCC AAC CCC TAC GAC GAT
ala ala pro ala ala glu thr leu asp arg arg ala ala leu pro asn pro tyr asp asp
121/41                                              151/51
CCC TTC TAC ACG ACG CCA TCC AAC ATC GGC ACG TTT GCC AAG GGC CAG GTG ATC CAA TCT
pro phe tyr thr thr pro ser asn ile gly thr phe ala lys gly gln val ile gln ser
181/61                                              211/71
CGC AAG GTG CCC ACG GAC ATC GGC AAC GCC AAC AAC GCT GCG TCG TTC CAG CTG CAG TAC
arg lys val pro thr asp ile gly asn ala asn asn ala ala ser phe gln leu gln tyr
241/81                                              271/91
CGC ACC ACC AAT ACG CAG AAC GAG GCG GTG GCC GAC GTG GCC ACC GTG TGG ATC CCG GCC
arg thr thr asn thr gln asn glu ala val ala asp val ala thr val trp ile pro ala
301/101                                             331/111
AAG CCC GCT TCG CCG CCC AAG ATC TTT TCG TAC CAG GTC TAC GAG GAT GCC ACG GCG CTC
lys pro ala ser pro pro lys ile phe ser tyr gln val tyr glu asp ala thr ala leu
361/121                                             391/131
GAC TGT GCT CCG AGC TAC AGC TAC CTC ACT GGA TTG GAC CAG CCG AAC AAG GTG ACG GCG
asp cys ala pro ser tyr ser tyr leu thr gly leu asp gln pro asn lys val thr ala
421/141                                             451/151
GTG CTC GAC ACG CCC ATC ATC ATC GGC TGG GCG CTG CAG CAG GGC TAC TAC GTC GTC TCG
val leu asp thr pro ile ile ile gly trp ala leu gln gln gly tyr tyr val val ser
481/161                                             511/171
TCC GAC CAC GAA GGC TTC AAA GCC GCC TTC ATC GCT GGC TAC GAA GAG GGC ATG GCT ATC
ser asp his glu gly phe lys ala ala phe ile ala gly tyr glu glu gly met ala ile
541/181                                             571/191
CTC GAC GGC ATC CGC GCG CTC AAG AAC TAC CAG AAC CTG CCA TCC GAC AGC AAG GTC GCT
leu asp gly ile arg ala leu lys asn tyr gln asn leu pro ser asp ser lys val ala
601/201                                             631/211
CTT GAG GGC TAC AGT GGC GGA GCT CAC GCC ACC GTG TGG GCG ACT TCG CTT GCT GAA TCG
leu glu gly tyr ser gly gly ala his ala thr val trp ala thr ser leu ala glu ser
661/221                                             691/231
TAC GCG CCC GAG CTC AAC ATT GTC GGT GCT TCG CAC GGC GGC ACG CCC GTG AGC GCC AAG
tyr ala pro glu leu asn ile val gly ala ser his gly gly thr pro val ser ala lys
721/241                                             751/251
GAC ACC TTT ACA TTC CTC AAC GGC GGA CCC TTC GCC GGC TTT GCC CTG GCG GGT GTT TCG
asp thr phe thr phe leu asn gly gly pro phe ala gly phe ala leu ala gly val ser
781/261                                             811/271
GGT CTC TCG CTC GCT CAT CCT GAT ATG GAG AGC TTC ATT GAG GCC CGA TTG AAC GCC AAG
gly leu ser leu ala his pro asp met glu ser phe ile glu ala arg leu asn ala lys
841/281                                             871/291
GGT CAG CGG ACG CTC AAG CAG ATC CGC GGC CGT GGC TTC TGC CTG CCG CAG GTG GTG TTG
gly gln arg thr leu lys gln ile arg gly arg gly phe cys leu pro gln val val leu
901/301                                             931/311
ACC TAC CCC TTC CTC AAC GTC TTC TCG CTG GTC AAC GAC ACG AAC CTG CTG AAT GAG GCG
thr tyr pro phe leu asn val phe ser leu val asn asp thr asn leu leu asn glu ala
961/321                                             991/331
CCG ATC GCT AGC ATC CTC AAG CAG GAG ACT GTG GTC CAG GCC GAA GCG AGC TAC ACG GTA
pro ile ala ser ile leu lys gln glu thr val val gln ala glu ala ser tyr thr val
1021/341                                            1051/351
TCG GTG CCC AAG TTC CCG CGC TTC ATC TGG CAT GCG ATC CCC GAC GAG ATC GTG CCG TAC
ser val pro lys phe pro arg phe ile trp his ala ile pro asp glu ile val pro tyr
1081/361                                            1111/371
CAG CCT GCG GCT ACC TAC GTC AAG GAG CAA TGT GCC AAG GGC GCC AAC ATC AAT TTT TCG
gln pro ala ala thr tyr val lys glu gln cys ala lys gly ala asn ile asn phe ser
1141/381                                            1171/391
CCC TAC CCG ATC GCC GAG CAC CTC ACC GCC GAG ATC TTT GGT CTG GTG CCT AGC CTG TGG
pro tyr pro ile ala glu his leu thr ala glu ile phe gly leu val pro ser leu trp
1201/401                                            1231/411
TTT ATC AAG CAA GCC TTC GAC GGC ACC ACA CCC AAG GTG ATC TGC GGC ACT CCC ATC CCT
phe ile lys gln ala phe asp gly thr thr pro lys val ile cys gly thr pro ile pro
```

Fig 1C

Prepro C.antarctica lipase A -> I-phase Translation

```
1261/421                                1291/431
GCT ATC GCT GGC ATC ACC ACG CCC TCG GCG GAC CAA GTG CTG GGT TCG GAC CTG GCC AAC
ala ile ala gly ile thr thr pro ser ala asp gln val leu gly ser asp leu ala asn
1321/441                                1351/451
CAG CTG CGC AGC CTC GAC GGC AAG CAG AGT GCG TTC GGC AAG CCC TTT GGC CCC ATC ACA
gln leu arg ser leu asp gly lys gln ser ala phe gly lys pro phe gly pro ile thr
1381/461
CCA CCT
pro pro
```

Fig 1C (cont.)

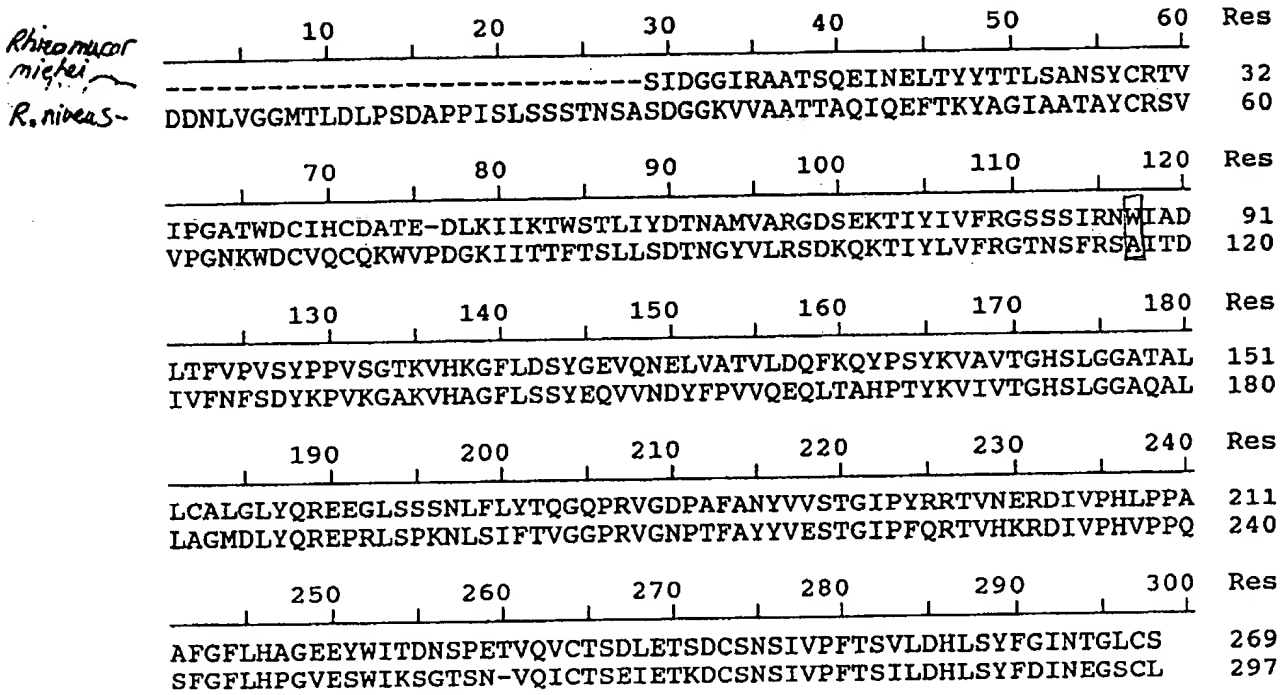
Rhizomucor miehei
R. niveus
10 20 30 40 50 60 Res
----------SIDGGIRAATSQEINELTYYTTLSANSYCRTV 32
DDNLVGGMTLDLPSDAPPISLSSSTNSASDGGKVVAATTAQIQEFTKYAGIAATAYCRSV 60
70 80 90 100 110 120 Res
IPGATWDCIHCDATE-DLKIIKTWSTLIYDTNAMVARGDSEKTIYIVFRGSSSIRNWIAD 91
VPGNKWDCVQCQKWVPDGKIITTFTSLLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITD 120
130 140 150 160 170 180 Res
LTFVPVSYPPVSGTKVHKGFLDSYGEVQNELVATVLDQFKQYPSYKVAVTGHSLGGATAL 151
IVFNFSDYKPVKGAKVHAGFLSSYEQVVNDYFPVVQEQLTAHPTYKVIVTGHSLGGAQAL 180
190 200 210 220 230 240 Res
LCALGLYQREEGLSSSNLFLYTQGQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPA 211
LAGMDLYQREPRLSPKNLSIFTVGGPRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQ 240
250 260 270 280 290 300 Res
AFGFLHAGEEYWITDNSPETVQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCS 269
SFGFLHPGVESWIKSGTSN-VQICTSEIETKDCSNSIVPFTSILDHLSYFDINEGSCL 297

Fig. 2

```
  1 MDDSVNTRYP ILLVHGLFGF DRIGSHHYFH GIKQALNECG ASVFVPIISA
 51 ANDNEARGDQ LLKQIHNLRR QVGAQRVNLI GHSQGALTAR YVAAIAPELI
101 ASVTSVSGPN HGSELADRLR LAFVPGRLGE TVAAALTTSF SAFLSALSGH
151 PRLPQNALNA LNALTTDGVA AFNRQYPQGL PDRWGGMGPA QVNAVHYYSW
201 SGIIKGSRLA ESLNLLDPLH NALRVFDSFP TRETRENDGM VGRFSSHLGQ
251 VIRSDYPLDH LDTINHMARG SAGASTR
```

Fig 3

	X		Y		
	42				? 89
A37916	TYGDEDCLYL	NIWVPQGRK.	..QVSRDLPV	MIWIYGGAFL	MGSGHGANFL
Lirtt	EVVTEDGYIL	GVYRIPHGKN	NSENIGKRPV	VYLQHGLIAS	AT..NWIANL
S07145	EVVTEDGYIL	EVNRIPYGKK	NSGNTGQRPV	VFLQHGLLAS	AT..NWISNL
Lidg	TNKNPNNFQT	LLPSDPSTIE	ASNFQTDKKT	RFTIHGFINK	GE.ENWLLDM
A34494	TNENPNNFQE	VA.ADSSSIS	GSNFKTNRKT	RFIIHGFIDK	GE.ENWLANV
A34671	TNENPNNYQI	ISATDPATIN	ASNFQLDRKT	RFIIHGFIDK	GE.EGWLLDM
Lipg	TNQNQNNYQE	LV.ADPSTIT	NSNFRMDRKT	RFIIHGFIDK	GE.EDWLSNI
A33553	GETNQ..GCQ	IRINHPDTLQ	ECGFNSSLPL	VMIIHGWSVD	GVLENWIWQM
A27442	KDESDRLGCQ	LRPQHPETLQ	ECGFNSSHPL	VMIIHGWSVD	GLLETWIWKI

	90				130
A37916	NNYLYDGEEI	ATRGNVIVVT	FNYRVGPLGF	LSTGDANLPG	NYGLRDQHMA
Lirtt	PNNSLAFMLA	DAGYDVWLGN	SRGNTWSRKN	VYYSPDSVEF	WAFSFDEMAK
S07145	PNNSLAFILA	DAGYDVWLGN	SRGNTWARRN	LYYSPDSVEF	WAFSFDEMAK
Lidg	CKNMFKVEE.	........VN	CICVDWKKGS	QTSYTQAANN	VRVVGAQVAQ
A34494	CKNLFKVES.	........VN	CICVDWKGGS	RTGYTQASQN	IRIVGAEVAY
A34671	CKKMFQVEK.	........VN	CICVDWKRGS	RTEYTQASYN	TRVVGAEIAF
Lipg	CKNLFKVES.	........VN	CICVDWKGGS	RTGYTQASQN	IRIVGAEVAY
A33553	VAALKSQPAQ	P.......VN	VGLVDWITLA	HDHYTIAVRN	TRLVGKEVAA
A27442	VGALKSRQSQ	P.......VN	VGLVDWISLA	YQHYAIAVRN	TRVVGQEVAA

	131				175
A37916	IAWVKRNI.A	AFGGDPNNIT	LFGESAGGAS	VSLQTLSPYN	K...GLIRRA
Lirtt	YDLPATINFI	VQKTGQEKIH	YVGHSQGTTI	GFIAFSTNPT	L..AKKIKTF
S07145	YDLPATIDFI	VKKTGQKQLH	YVGHSQGTTI	GFIAFSTNPS	L..AKRIKTF
Lidg	MLSMLS...A	NYSYSPSQVQ	LIGHSLGAHV	AGEAGSRTPG	...LGRITGL
A34494	FVEFLQ...S	AFGYSPSNVH	VIGHSLGAHA	AGEAGRRTNG	T..IGRITGL
A34671	LVQVLS...T	EMGYSPENVH	LIGHSLGSHV	AGEAGRRLEG	H..VGRITGL
Lipg	FVEVLK...S	SLGYSPSNVH	VIGHSLGSHA	AGEAGRRTNG	T..IERITGL
A33553	LLRWLE...E	SVQLSRSHVH	LIGYSLGAHV	SGFAGSSIGG	THKIGRITGL
A27442	LLLWLE...E	SMKFSRSKVH	LIGYSLGAHV	SGFAGSSMGG	KRKIGRITGL

	176				220
A37916	ISQSGVALSP	WVIQKN....	..PLFWAKKV	AEKVGCPVGD	AARMAQCLKV
Lirtt	YALAPVATVK	YTQSPLKKIS	FIPTFLFKLM	FGKKMFLPHT	YFDDFLGTEV
S07145	YALAPVATVK	YTKSLINKLR	FVPQSLFKFI	FGDKIFYPHN	FFDQFLATEV
Lidg	DPVEASFQGT	PEEVRLD...	..PTDADFVD	VIHTDAAPLI	PFLGFGTSQQ
A34494	DPAEPCFQGT	PELVRLD...	..PSDAKFVD	VIHTDGAPIV	PNLGFGMSQV
A34671	DPAEPCFQGL	PEEVRLD...	..PSDAMFVD	VIHTDSAPII	PYLGFGMSQK
Lipg	DPAEPCFQGT	PELVRLD...	..PSDAKFVD	VIHTDAAPII	PNLGFGMSQT
A33553	DAAGPLFEGS	APSNRLS...	..PDDASFVD	AIHTFTREHM	GLSVGIK.QP
A27442	DPAGPMFEGT	SPNERLS...	..PDDANFVD	AIHTFTREHM	GLSVGIK.QP

z = FLAP region

		221				270
1 —	A37916	TDPRALTLAY	KVPLAGLEYP	MLHYVGFVPV	IDGDFIPADP	INLYANAADI
2 —	Lirtt	CSREVLDLLC	SNTLFIFCGF	DKKNLNVSRF	DVYLGHNPAG	TSVQDFLHWA
3 —	S07145	CSREMLNLLC	SNALFIICGF	DSKNFNTSRL	DVYLSHNPAG	TSVQNMFHWT
4 —	Lidg	MGHLDFFPNG	GEEMPGCKKN	ALSQIVNLDG	IWEGTRDFVA	CNHLRSYKYY
5 —	A34494	VGHLDFFPNG	GVEMPGCKKN	ILSQIVDIDG	IWEGTRDFAA	CNHLRSYKYY
6 —	A34671	VGHLDFFPNG	GKEIPGCQKN	ILSTIVDING	IWEGTRNFAA	CNHLRSYKYY
7 —	Lipg	VGHLDFFPNG	GKQMPGCQKN	ILSQIVDIDG	IWEGTRDFVA	CNHLRSYKYY
8 —	A33553	IGHYDFYPNG	GSFQPGCHFL	ELYRHIAQHG	FNAITQTIK.	CSHERSVHLF
9 —	A27442	IAHYDFYPNG	GSFQPGCHFL	ELYKHIAEHG	LNAITQTIK.	CAHERSVHLF

Fig. 4

A A L P N P Y D D P F Y T T P S N I

N-terminal sequence of C.antartica LF 058 lipase component A

AACCCATACGACGACCC NOR 440
T C T T T
G
T

GCTGCTCTGCCTAACCCTTACGACGACCCTTTCTACACCACCCC NOR 438 ; Guess positions indicated
T T C

Fig. 5

NZAS-0014715

```
   1  TCGAACGATG CGAGTGTCCT TGCGCTCCAT CACGTCGCTG CTTGCGGCGG
  51  CAACGGCGGC TGTGCTCGCG GCTCCGGCGG CCGAGACGCT GGACCGACGG
 101  GCGGCGCTGC CCAACCCCTA CGACGATCCC TTCTACACGA CGCCATCCAA
 151  CATCGGCACG TTTGCCAAGG GCCAGGTGAT CCAATCTCGC AAGGTGCCCA
 201  CGGACATCGG CAACGCCAAC AACGCTGCGT CGTTCCAGCT GCAGTACCGC
 251  ACCACCAATA CGCAGAACGA GGCGGTGGCC GACGTGGCCA CCGTGTGGAT
 301  CCCGGCCAAG CCCGCTTCGC CGCCCAAGAT CTTTTCGTAC CAGGTCTACG
 351  AGGATGCCAC GGCGCTCGAC TGTGCTCCGA GCTACAGCTA CCTCACTGGA
 401  TTGGACCAGC CGAACAAGGT GACGGCGGTG CTCGACACGC CCATCATCAT
 451  CGGCTGGGCG CTGCAGCAGG GCTACTACGT CGTCTCGTCC GACCACGAAG
 501  GCTTCAAAGC CGCCTTCATC GCTGGCTACG AAGAGGGCAT GGCTATCCTC
 551  GACGGCATCC GCGCGCTCAA GAACTACCAG AACCTGCCAT CCGACAGCAA
 601  GGTCGCTCTT GAGGGCTACA GTGGCGGAGC TCACGCCACC GTGTGGGCGA
 651  CTTCGCTTGC TGAATCGTAC GCGCCCGAGC TCAACATTGT CGGTGCTTCG
 701  CACGGCGGCA CGCCCGTGAG CGCCAAGGAC ACCTTTACAT TCCTCAACGG
 751  CGGACCCTTC GCCGGCTTTG CCCTGGCGGG TGTTTCGGGT CTCTCGCTCG
 801  CTCATCCTGA TATGGAGAGC TTCATTGAGG CCCGATTGAA CGCCAAGGGT
 851  CAGCGGACGC TCAAGCAGAT CCGCGGCCGT GGCTTCTGCC TGCCGCAGGT
 901  GGTGTTGACC TACCCCTTCC TCAACGTCTT CTCGCTGGTC AACGACACGA
 951  ACCTGCTGAA TGAGGCGCCG ATCGCTAGCA TCCTCAAGCA GGAGACTGTG
1001  GTCCAGGCCG AAGCGAGCTA CACGGTATCG GTGCCCAAGT TCCCGCGCTT
1051  CATCTGGCAT GCGATCCCCG ACGAGATCGT GCCGTACCAG CCTGCGGCTA
1101  CCTACGTCAA GGAGCAATGT GCCAAGGGCG CCAACATCAA TTTTTCGCCC
1151  TACCCGATCG CCGAGCACCT CACCGCCGAG ATCTTTGGTC TGGTGCCTAG
1201  CCTGTGGTTT ATCAAGCAAG CCTTCGACGG CACCACACCC AAGGTGATCT
1251  GCGGCACTCC CATCCCTGCT ATCGCTGGCA TCACCACGCC CTCGGCGGAC
1301  CAAGTGCTGG GTTCGGACCT GGCCAACCAG CTGCGCAGCC TCGACGGCAA
1351  GCAGAGTGCG TTCGGCAAGC CCTTTGGCCC CATCACACCA CCTTAGTCGG
1401  CGAGCACTTT CGGATGTTCC GCTACAGTTC CCACGGTCGA TGCCGACCTT
1451  CAGAGATCAC GAGTGACCTC GGG
```

Fig 6

Table I

Amino acid composition of C. antartica lipase A (CALIP)

	Deduced from DNA sequence	By amino acid analysis (MC)
Ala	50	47
Arg	9	9
Asp/AsN	35	36
Cys	4	4
Gln/GlN	35	36
Gly	28	31
His	6	6
Ile	26	24
Leu	29	30
Lys	17	17
Met	2	3
Phe	20	19
Pro	33	33
Ser	26	27
Thr	27	28
Trp	5	4
Tyr	18	16
Val	27	26

Fig. 7

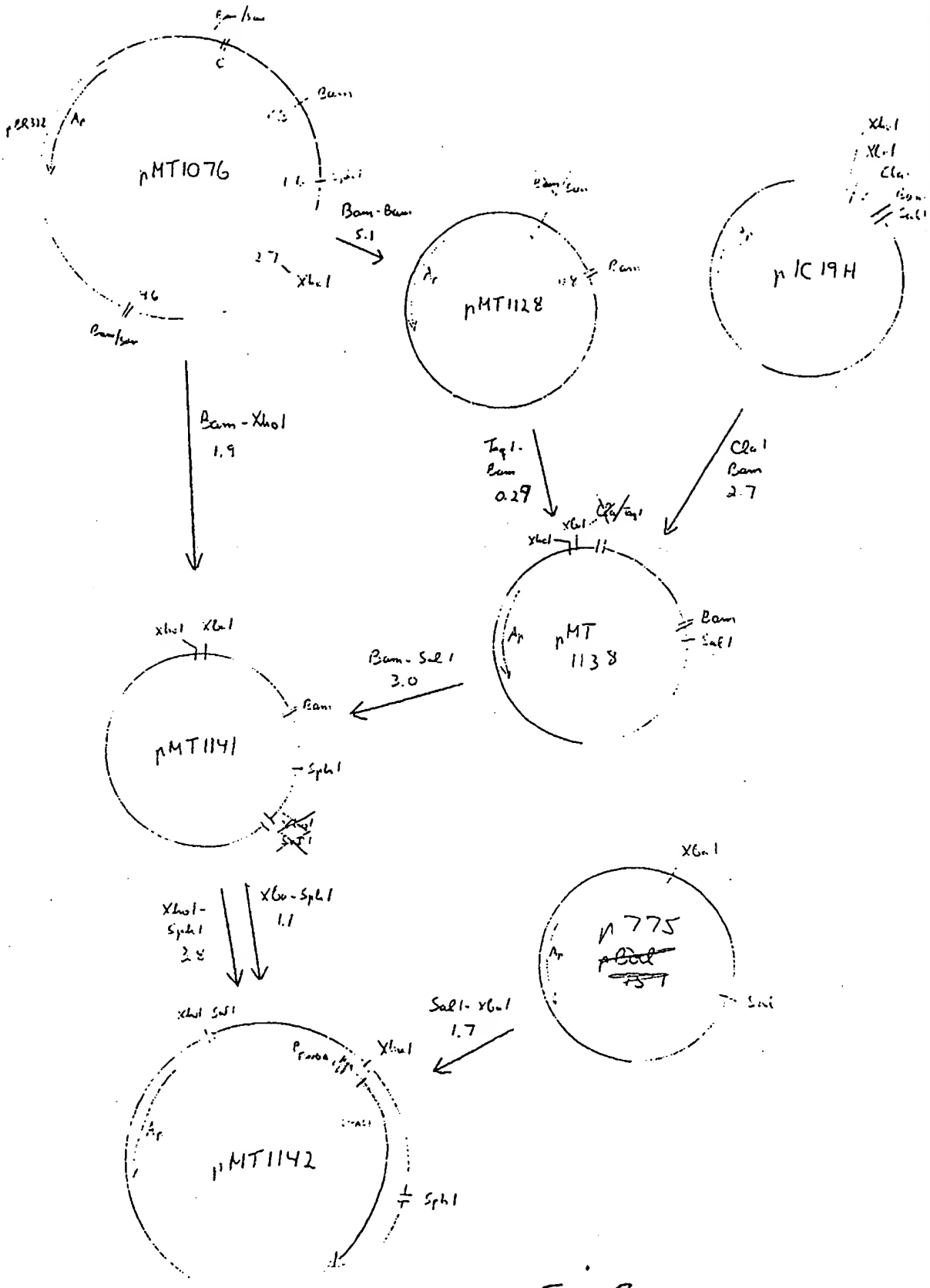
pMT1076
pER332
Ap
Bam
Bam/Bam
Bam-Bam
5.1
2.7
XbaI
4.6
pMT1128
Ap
Bam
pIC19H
XbaI
ClaI
Bam-XhoI
1.9
TaqI-Bam
0.29
ClaI-Bam
2.7
XbaI
ClaI/TaqI
pMT
1138
Ap
Bam
SalI
XhoI
XbaI
pMT1141
Bam
SphI
Bam-SalI
3.0
XhoI-SphI
2.8
XbaI-SphI
1.1
XbaI
p775
Ap
SacI
SalI-XbaI
1.7
XhoI
SalI
pMT1142
Ap
XhoI
SphI

Fig. 8

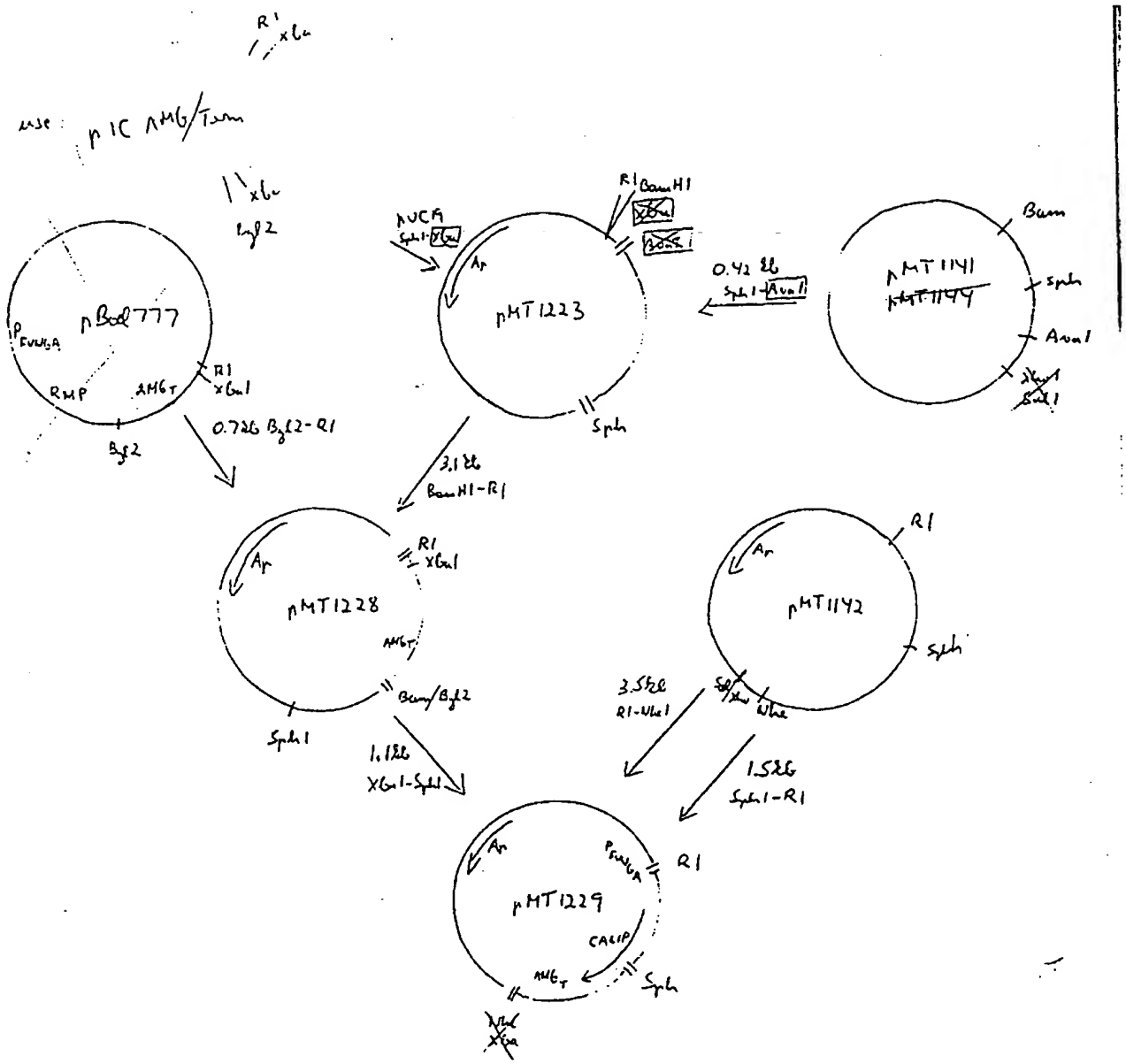

Fig 9: Construction of expression plasmid for C. antarctica lipase A

[redacted]